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# BIOMARKERS OF EXPOSURE TO TOXIC SUBSTANCES

**Volume I: Global Experimental Design: Biomarker Discovery for Early Prediction of Organ-Selective Toxicity**

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The work described in the following five reports was initiated to discover, down-select, and prevalidate biomarkers of toxic substance effects within the warfighter before health and operational performance are reduced. Using the emerging biotechnologies of toxicogenomics, proteomics, metabonomics, and computer modeling, this effort focused on identification of organ-selective toxic effects to militarily relevant chemical exposure(s) before significant decrement jeopardizes mission performance and detrimental effects are manifested. Results indicate blood and urine samples could be used to detect very low level changes (using either simple statistics ($p<0.05$) or computer-based multi-dimensional statistical analysis) in either one family or combined molecules from families of messenger RNA, proteins, or metabolites post-exposure to several known toxic chemicals.

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## FORWARD

This research program is documented in a final technical report comprised of five volumes. Volume 1 provides a global overview of the entire effort. Volumes II-IV provide the technical details of the three approaches (genomics, proteomics, and metabonomics) used to identify the relevant biomarkers of toxic effects. Volume V describes the effort to perform pre-validation of the identified biomarkers. Figure 1 shows this technical report structure.

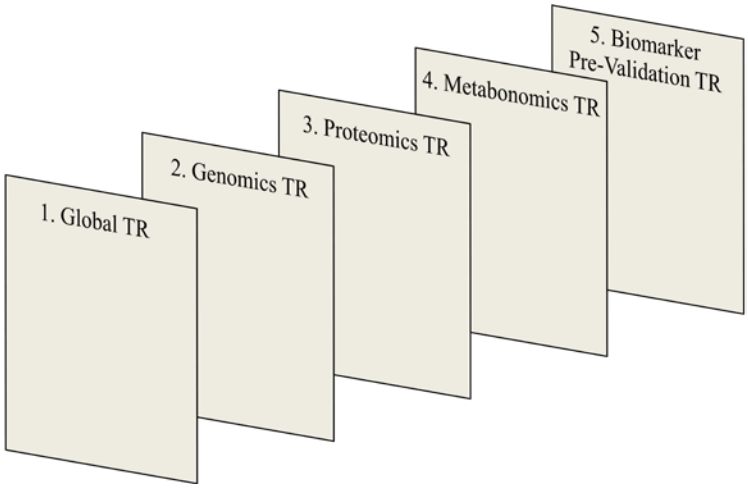


**Figure 1: Technical Report Volume Order**

Volume I contains the experimental design, explains how the needs of the warfighter led to conducting this research effort, the reasoning behind the specific analysis method and biomarker selections, and the manner in which the specimens were collected. The sample analysis is captured in Volumes II, III and IV (Genomics, Proteomics, and Metabonomics). The three analytical and investigational approaches were conducted in parallel and fed data into the fifth report (Biomarker Pre-validation) as depicted in Figure 2.

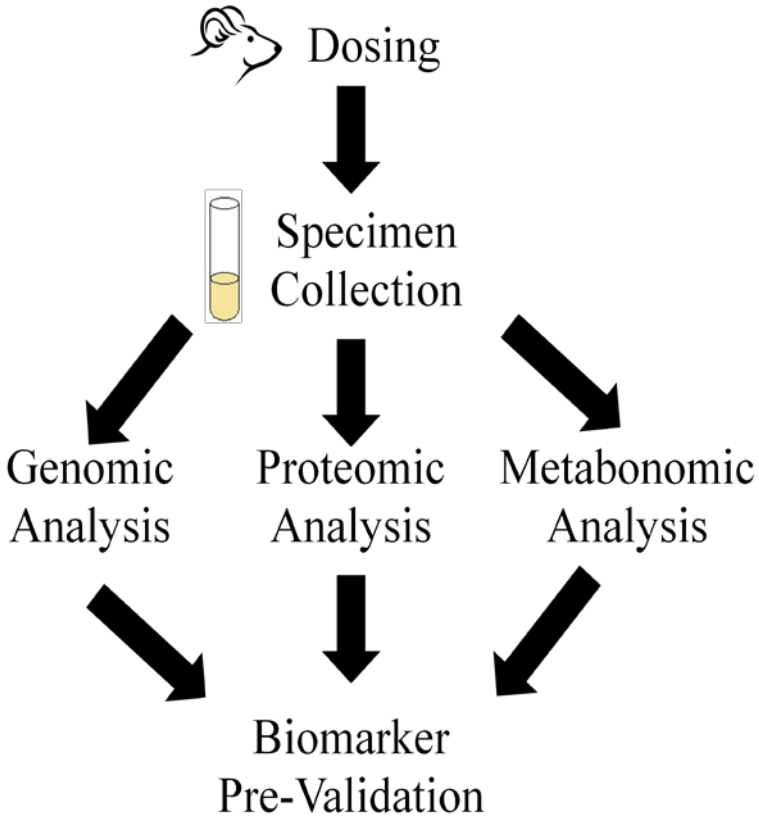


**Figure 2: Work Unit Investigational Overview**

Over 80 Department of Defense civilians, contractors, and military contributed in the research spanning five years.

## PREFACE

This research was accomplished at the Applied Biotechnology Branch, Human Effectiveness Directorate of the 711th Human Performance Wing (711 HPW/RHPB) of the Air Force Research Laboratory, Wright-Patterson AFB, OH, under Dr. John J. Schlager, Branch Chief. This work unit report was written for AFRL Work Unit 7184D405.

All studies involving animals were approved by the Wright-Patterson Institutional Animal Care and Use Committee, and were conducted in a facility accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care, International, in accordance with the *Guide for the Care and Use of Laboratory Animals* (1996).

## SUMMARY

The work described in the following five volumes was initiated to discover, down-select, and pre-validate biomarkers of toxic substance effects within the warfighter before experiencing reduced health and operational performance. Using the emerging biotechnologies of toxicogenomics, proteomics, metabonomics, and computer modeling, this effort focused on identification of organ-selective toxic effects to militarily relevant chemical exposure(s) before significant decrement immediately jeopardizes mission performance and future detrimental effects are manifested. Results indicate blood and urine samples could be used to detect low level changes (using either simple statistics ($p<0.05$) or computer-based multi-dimensional statistical analysis) in either one family or combined molecules from families of messenger RNA, proteins, or metabolites post-exposure to several known toxic chemicals.

# 1. INTRODUCTION

The Defense Intelligence Report DI-1816-8-99 states, “Personnel deployed in support of missions ranging from war to operations other than war may be exposed to harmful chemicals as a result of industrial accidents, sabotage, or the intentional or unintentional actions of enemy or friendly forces.” Therefore, an increasingly important issue in force protection is the toxicology associated with potential chemical exposures at uncharacterized deployed sites. Furthermore, it must be assumed that these uncharacterized environments contain contaminants that represent chemical mixtures and complex exposure patterns. Rapid risk assessment of poorly characterized or uncharacterized environments, and those from chemical mixtures exposure, requires significant study for development of new testing methods. Since deployed personnel may be exposed to multiple dynamic environmental hazards during deployment as a result of industrial accidents, intentional or unintentional activities of various forces (enemy or friendly) or sabotage, rapid assessment of field-forward methodology for force readiness and health monitoring is required. The development and application of biomarkers isolated from these biotechnologies will prevent serious injury to deployed warfighters exposed to toxic substances and environments. Development of novel human markers of organ-selective exposure and the monitoring methodologies that provide real-time detection of potential toxic injury will minimize mission degradation due to environmentally related adverse health effects. This information can provide the field commanders with fast and accurate toxicity information for quicker alternative site selection decisions, minimizing individual deployment time in hazardous environments and identifying proper levels of protective equipment.

U.S. military forces deploy to many regions of the world that may lack the environmental controls found in more developed nations. Operational fixed sites such as airbases, seaports, and major logistic facilities are located in areas requiring access to fuel, electricity, roads, and other infrastructure that are also necessary for industry production facilities of the emerging nations. Therefore, there is a high probability that facilities operated by U.S. warfighters would be contaminated with toxic materials and chemicals. At present, there is no device to monitor warfighters to ensure they are protected from toxic substances in their working or living environment. Current methods of determining toxic exposures to the warfighter are not adequate to prevent serious health effects such as were induced by Herbicide Orange in Vietnam, or to predict and prevent low-level exposures that could have immediate performance or delayed health impacts. Simple chemical analysis of the environment is not sufficient to predict the effect of the myriad of combinations of diverse toxic substances at multiple exposure concentrations. To prevent future medically unexplained physical symptoms as potential injuries, it is necessary to devise a system set that will identify exposure of personnel to very low, subtoxic concentrations of harmful substances before they affect the individual. The first major challenge will be to determine which changes in the data rich biotechnologies of transcriptional genomics, proteomics, and metabonomics are indicative of a detrimental effect on human health compared to an adaptive effect that does not impact warfighter well-being and effectiveness (recovery) in response to hazardous exposure. Also, a significant challenge is the successful use of relatively noninvasive identification methods to include collection of urine, blood, or epithelial cells for isolation and identification of critical health events.

Presently, measurable endpoints of chemical toxicity can range from histopathological changes to biochemical activities of various cellular enzymes/constituents, to the expression of

specific genes/proteins/metabolites, and more recently, to the global gene/protein/metabolite expression profiles in target organs or biofluids. It is widely believed that gene, protein and metabolite expression changes are more accurate, reliable, sensitive, and informative as quantitative endpoints than the traditional clinical chemistry toxicity endpoints. Genomic, proteomic and metabonomic technologies are ideal tools to measure the changes in gene, protein and metabolite expression resulting from systemic toxicant exposure. Understanding the cellular response to chemical exposure at the molecular level will not only facilitate the elucidation of the mechanism of chemical toxicity, but also allow accurate prediction of chemical toxicity and phenotypic outcome. Ultimately, it will lead to the identification of novel biomarkers for rapid monitoring and prediction of health hazards associated with chemical exposure, as well as pinpointing gene, protein and metabolic pathway targets for nutritional and pharmaceutical prophylactic and therapeutic intervention. Therefore, it is imperative that the methodologies indicated below be further developed so that more accurate predictions of impending toxicity or disease can be formed.

### 1.1. Genomics

Differentially expressed genes in various types of samples (e.g. blood, target organ tissues, etc.) can be compared by conducting gene expression profiling studies. These studies provide cell/organ state information with respect to regulatory mechanisms and phenotypic activity of genes that comprise an organism's genome. Expression profiling techniques have become widely used in research and diagnostics to uncover disease pathways (Schena et al., 1995). DNA microarray analysis has become the most accepted technique among gene expression profiling techniques to initially monitor differential expression levels (Eisen et al., 1998). DNA microarray analysis allows for profiling expression of tens of thousands of gene transcripts in parallel for high through-put screening and is relatively easy to use.

Construction of gene microarrays for the quantitative assessment of transcriptional activity of tens of thousands of genes has resulted from the availability of gene sequences and physical clones of the isolated sequence from the coding region of these genes (Schena et al., 1995). A recent review of DNA microarray technology and its applications to mechanistic and predictive toxicology can be found in the literature (Pennie et al., 2000). The application of toxicogenomics, the study of how genomes respond to environmental stressors or toxicants, will be highly beneficial in providing rapid profound assessment of toxicity in controlled laboratory exposure studies. Analogous to the area of drug discovery, increased mechanistic toxicity information will provide more accurate human risk assessments and direct research to biomarker pathways for identifying the best methods for monitoring human health.

### 1.2. Proteomics

Proteomics refer to the study of all the proteins that can be synthesized from an organism's genome within a certain expressed phenotype (e.g. normal verses altered). The determination of protein function is a major challenge of the post-genomic era. The speed at which target proteins can be isolated and identified will be the rate-limiting step in the establishment of proteomics as a useful diagnostic tool. The word "proteomics" was first reported in the literature by Wasinger et al. (1995) and refers to the "total protein complement of a genome." Therefore, proteomics is the study of proteomes and involves the measurement and

analysis of proteins expressed by a cell at any given time. A review of proteome research can be found in the literature (Humphery-Smith et al., 1997).

Functional information for all cellular proteins will eventually be provided by proteomic studies. Currently, proteomic research is being driven by cell-mapping and protein expression strategies. With respect to the topic of medical surveillance technology, the protein expression strategy is the most viable. This strategy is concerned with monitoring global expression of large numbers of proteins within a cell or tissue, and quantitatively identifies pattern changes resulting from hazardous chemical or material exposures. The goal of expression profiling is to generate "protein fingerprints" that may provide insight into novel biomarkers of disease or toxicity.

### 1.3. Metabonomics

Metabonomics is defined as "the quantitative measurement of the time-related multiparametric metabolic response of living organisms to pathophysiological stimuli or genetic modification" (Nicholson et al., 1999). This term is derived from the Greek roots "meta" (change) and "nomos" (regularity and order); referring to the ability of chemometric models to classify changes in metabolism (Lindon et al., 2004). This biotechnology was pioneered by Jeremy Nicholson, Elaine Holmes and John Lindon in the late 1990s at the Imperial College in London (Nicholson et al., 1999). The field of metabonomics is concerned with the study of fixed cellular and biofluid concentrations of endogenous metabolites, as well as dynamic metabolite fluctuations, exogenous species, and molecules that arise from chemical rather than enzymatic processing (Lindon et al., 2003).

Metabonomics is an approach used to characterize the metabolic profile of a specific tissue or biofluid. Biofluids are typically used in metabonomic studies because can be easily obtained either non-invasively (urine) or minimally invasively (blood). However, other *in vivo* biofluids such as saliva, cerebrospinal fluid, bile, and seminal fluid, as well as *in vitro* biofluids such as cell culture supernatants and tissue extracts, can also be used. Metabonomics is an attractive approach to the study of time-related metabolic responses to pathophysiological processes because biological and chemical agents, or drugs, cause perturbations in the concentrations and fluxes of endogenous metabolites involved in critical cellular pathways. In other words, cells respond to toxic insult or other stressors by altering their intra and/or extracellular environment in an attempt to maintain a homeostatic intracellular environment. This metabolic alteration is expressed as a "fingerprint" of biochemical perturbations that are characteristic of the type and target of a toxic insult or disease process. These alterations are often seen in the urine as changes in metabolic profile in response to toxicity or disease as the body attempts to maintain homeostasis by eliminating substances from the body. Subtle responses to toxicity or disease under conditions of homeostasis also result in altered biofluid composition. A recent article by Don Robertson (2005) provides an excellent review of the use of metabonomics in toxicology.

A frequent misconception is that metabonomics is based primarily on nuclear magnetic resonance spectroscopy (NMR)-derived data. In theory, any technology that has the capacity to generate comprehensive metabolite measurements can be used for metabonomics. The most common analytical platforms used today in metabonomics are proton NMR and mass spectroscopy (MS) coupled to liquid chromatography (LC) and gas chromatography. The

advantages of NMR-based metabonomics include nondestructive analysis, analysis of intact tissues, molecular structure analysis, and quantitative small-molecule analysis. The non-selectivity, lack of sample bias and reproducibility of NMR (Keun et al., 2002) is of critical importance when considering toxicological screening applications. Changes in NMR-derived urinary metabolite levels have proven to be a sensitive indicator of chemical-induced toxicity (Robertson et al., 2000; Holmes and Shockcor, 2000; Waters et al., 2002; Nicholson, et al., 2002). MS offers the ability to detect chemical classes not detected by NMR (i.e. sulfates), and the capability to detect lower abundance metabolites with little sample processing (Dunn and Ellis 2005). This is of critical importance if one is searching for novel biomarkers of toxicity or disease. Urinary metabolite analysis using LC-MS has also been used to profile chemical-induced toxicity (La et al., 2005). It is clear to see that MS is complimentary to NMR data and facilitates metabolite identification.

## 1.4. Chemical Selection

The objective of the present study was to identify organ-selective toxicity biomarkers for two organ systems in a rat animal model. The two organ systems selected were kidney and liver. Although some initial work with a liver toxicant (alpha-naphthylisothiocyanate; ANIT) was performed under an earlier protocol (data not shown), the data presented in this final report is concerned with changes in gene transcript, protein and metabolite levels associated with kidney specific toxicity in rats following exposure to rationally selected chemicals. Four chemicals were chosen (D-serine, puromycin, hippuric acid and amphotericin B) that targeted different functional regions of the kidney (proximal tubules, distal tubules, glomerulus and medulla, respectively). Profile changes, after additional testing and validation, may be proven to be sensitive and reliable biomarkers for acute organ-specific regional toxicity/damage in human population. A summary of the effects of the selected chemicals on the kidney is presented below.

### 1.4.1. D-Serine

D-serine is a normal plasma constituent in humans and contributes 0.5% to 3% to total plasma in normal humans. In patients with elevated serum creatinine levels, D-serine is present up to 23%. The increase of plasma D-serine concentrations in chronic renal failure indicates that the kidney keeps the plasma D-serine at low levels (Bruckner and Hausch, 1993; Nagata et al., 1992). D-serine is reabsorbed in the *pars recta* region of the rat proximal tubule and subsequently metabolized by D-aminoacid oxidase (D-AAO), to produce α–keto acid plus ammonia and hydrogen peroxide (Silbernagl et al., 1999; Pilone, 2000). Other findings suggest that metabolism of D-serine by D-AAO is responsible for the development of toxicity in the kidney (Maekawa et al., 2005). At high doses, however, D-serine causes selective necrosis to the *pars recta* region of the renal proximal tubules in the rat (Ganote et al., 1974). The damage is accompanied by proteinuria, glucosuria and aminoaciduria (Ganote et al., 1974; Williams et al., 2003). The process of renal toxicity is typically initiated by toxic injury to tubular epithelial cells in various segment of the nephron or by injury to specific cell types in the glomerulus (Amin et al., 2004). The initial injury is often followed by cellular proliferation that attempts to restore normal function of the kidney (Toback, 1992).

### 1.4.2. Puromycin

Puromycin aminonucleoside (PA) is an antibiotic produced in soil by the organism *Streptomyces alboniger* (Pierce et al., 1979). In the past, PA was used as an antineoplastic because of its ability to interfere with protein synthesis (Nathans, 1967). Puromycin blocks protein synthesis *in vitro* and *in vivo*, and its action appears to occur at the stage of formation of the polypeptide chain at the ribosomal site. Puromycin is active against a broad range of organisms that cause necrosis of glomerular podocytes, resulting in a severe glomerulopathy (Grond et al., 1988). Severe proteinuria results from the loss of the glomerular barrier to protein filtration, and tubular injury occurs secondary to the formation of proteinaceous casts in the proximal tubules (Ryan, 1986). Puromycin-induced nephrosis in rats provides a useful model for studying the pathogenesis of severe proteinuric conditions. Puromycin induces alterations in glomerular permselectivity and morphological changes as early as 24 h after a single intraperitoneal injection (Olson et al., 1981). Micropuncture experiments have clearly shown the glomerular origin of the proteinuria induced by PA (Landwehr et al., 1977; Oken et al., 1981).

### 1.4.3. Amphotericin B

Amphotericin B (AmpB) is an amphipathic fermentation product of the Gram-positive bacterium, *Streptomyces nodosus* that targets the distal tubules of the kidney. The major drawback to the use of AmpB is its insolubility in water. AmpB, a polyene macrolide antifungal, is formulated as a micelle suspension and is an effective broad spectrum agent in the treatment of systemic fungal infections (Polak, 2003; Meyer, 1992). However, AmpB use is often limited by the development of nephrotoxicity manifested by renal vasoconstriction with a significant decrease in glomerular filtration rate and renal plasma flow as well as by renal potassium and magnesium wasting (Harbarth et al., 2001; Wasan et al., 1998; Wasan and Conklin, 1997). The mechanism of action of AmpB is believed to be exerted through its interaction with ergosterol of fungal cells. This causes pores and channels to form within the fungal membrane and results in leaky fungal cell membranes potentially causing cell lysis and death. Although AmpB tends to bind to the fungal sterol preferentially, it has also been shown to bind to mammalian cholesterol (Brajtburg et al., 1990; Vertut-Croquin et al., 1983). Despite its high potential to induce dose-dependent kidney toxicity, AmpB remains the drug of choice for the treatment of severe systemic fungal infections (Khoo et al., 1994; Warnock, 1991; Gallis et al., 1990).

### 1.4.4. Hippuric Acid

Hippuric acid (HA), also known as n-benzoylglycine or benzoyl amidoacetic acid, is a uremic toxin that also targets the distal tubules of the kidney and accumulates in the plasma of patients with uremia due to chronic renal failure (Vanholder et al., 2003). Hippuric acid also accelerates the renal damage associated with chronic renal failure (Satoh et al., 2003). In addition, HA plays a role in a variety of pathological conditions such as stimulation of ammoniagenesis (Dzurik et al., 2001), inhibition of both plasma protein binding (Sakai et al., 1995) and organic anion secretion by the kidney (Boumendil-Podevin et al., 1975) and inhibition of glucose utilization in muscles (Spustova et al., 1987, 1989). Furthermore, serum and cerebrospinal fluid concentrations of HA correlate positively with neurophysiological indices

suggesting HA induction of neurological symptoms (Schoots et al., 1989). Hippuric acid itself does not have a direct biological function, but its formation in the liver results from detoxification of benzoic acid. Hippuric acid is the glycine conjugate of benzoic acid. In addition to the liver, HA is also formed by this reaction in the kidney (Wan and Riegelman, 1972; Kao et al., 1978; Poon and Pang, 1995) and in the intestine (Strahl and Barr, 1971). Glycine conjugation is catalyzed by benzoyl-Co A synthetase and benzoyl-Co A-glycine *N*-acyltransferase in the matrix of mitochondria (Gatley and Sherratt, 1977). Hippuric acid production is greatly increased following consumption of benzoic acid (e.g. foods containing benzoic acid preservatives) or substances which generate benzoic acid during intermediate metabolism (e.g. foods high in the polyphenols chlorogenic acid, quinic acid and caffeic acid). It appears in variable concentrations in urine and at much lower concentrations in plasma. Active tubular secretion is the primary route for elimination of HA from the plasma via the kidney, and functional failure of this system causes accumulation of HA in blood (Tsutsumi et al., 1999).

### 1.5. Biomarker Pre-validation

Once data obtained from disease/toxin models using RHPB 'integrated Omics'combined with bioinformatic analyses have identified potentially novel biomarkers, further quantitative testing must be completed in both animal and human model systems. These pre-validation and validation studies for proteins are usually accomplished by the development of ELISAs (enzyme-linked immunosorbent assay) to the biomarker presumed to be indicative of a given condition. The ELISA is an antibody-based assay in which a capture element (the primary antibody) selectively binds a given protein (biomarker) in a sample solution. The primary antibody has been developed using the purified biomarker protein which as an injected antigen, elicits an immunological response. This response permits the purification of the primary antibody using serum from the antigen- injected animal. A secondary antibody, binding the same protein molecule but at a different region (or epitope) is used for detection. These 'detection' antibodies may have chemicals or enzymes conjugated to the antibody or are a target for a third antibody/substrate. There are several different types of ELISAs, but with careful preparation and selection, all should detect a biomarker at nanogram to picogram per ml in fluids such as urine and serum. RNA isolated from peripheral blood mononuclear cells (PBMCs) or tissue may also be utilized as biomarkers using DNA based capture elements. In essence, with some technical changes, this technique is similar to ELISAs for proteins but with the hybridization of strands providing selectivity rather than protein/antibody binding.

It is important to recognize that until complete and definitive human validation studies have been accomplished, the biomarker can only be considered a 'potential' or 'pre-validated' marker. Biomarker validation studies are an expensive and extensive process, involving standardization of the assay/reagents and statistical analysis of test data for such parameters as sensitivity, specificity, and testing limits (analytical validation) (Lee et al., 2006, Rifai et al, 2006, Colburn et al., 2003). Final validation of the clinical ELISA involves confirmation of the utility and reproducibility of the test in human clinical trials (clinical validation). RHPB, as part of the biomarker discovery process, conducts research up to and including biomarker pre-validation.

### 1.6. Field Detection Using Biomonitor Devices

While the use of a clinical ELISA is well established, cutting edge detection elements and microfluidic fabrication are being used to develop small, point-of-care (POC) devices that may revolutionize both clinical and home care. There are numerous approaches for detection in these prototypes, ranging from Raman spectrophotometry (Grubisha et al, 2003), microcantilevers (Wu et al., 2001), ellipsometry (Ostroff et al., 1998), to hydragels (Carrigan et al., 2005). Each technique has strengths and weaknesses, but they all center around the use of antibodies as capture elements and thus are subject to inherent stability limitations. There are currently several marketed biomarker POC devices (Roche Diagnostics, Cardiac proBNP) and this strong need for home POCs is predicted to generate revenues of over $2.4 billion by 2012 in the US alone (Newswire, 2006). Thus, field use of well characterized toxicity biomarkers is best optimized by co-development of a small reader capable of detection and analysis of multiplexed assays. Ultimately, the unique biomarkers discovered during this research will be validated and incorporated into a POC device to provide on-site, real time personnel monitoring, in fulfillment of "Biotechnology for Near-Real-Time Predictive Toxicology" requirements.

## 2. METHODS

### 2.1. Animals

Male Fischer 344 rats (200-225 gram) purchased from Charles River Laboratories with surgically implanted jugular catheters were housed in Building 838. On the seventh day after arrival, 0.8 mL blood was taken via the jugular catheter using a 20-23 G needle to serve as the baseline for clinical chemistry analysis. At the end of the 14-day quarantine/acclimation period, the animals (30) were randomly assigned to a control or one of five dose groups (n = 5/group) and housed individually in metabolism cages. Food (Purina Certified Rat Chow # 5002) and water was available for all animals *ad libitum*. The housing environment was maintained on a 12 hour light-darkness cycle at 25 °C, and all animals were examined by Vivarium personnel twice daily. Urine samples were collected cold using plastic 50 mL conical tubes containing 1.0 mL of 1% sodium azide maintained at 6-10°C using I-Cups (Bioanalytical Systems, Inc.; stored at -80°C prior to use) 24 h prior to dosing and daily thereafter depending on the experimental design. Blood samples were collected in non-additive red top clot tubes (Becton-Dickinson) and serum was recovered by centrifugation at 2,500 x g at 25°C for 10 min. Post-exposure blood samples (0.8 mL) were collected on Day 1 (4-Day study), Day 1 and 4 (7-Day study) and Day 1, 4, 7 and 10 (14-Day study), and at termination of the study. Rats were euthanized by $CO_2$ overdose at study termination and additional tissues were collected for histopathology assessments. Tissue samples harvested for histopathologic analysis were collected immediately postmortem and fixed by immersion in 10% neutral-buffered formalin, routinely processed and embedded in paraffin; 4 to 6 micron sections were cut for hematoxylin and eosin staining according to standard procedure. Additionally, urine and blood samples were analyzed by liquid chromatography/mass spectrometry (LC/MS; positive or negative ion mode) and nuclear magnetic resonance (NMR; 600 mHz) spectroscopy. The results of these analyses were used to

guide the selection of appropriate doses and time period for the subsequent time-series experiment for genomic and proteomic studies (see below).

### 2.2. Dosing

After obtaining animal weights, rats were dosed with nephrotoxic agents dissolved in normal saline by intraperitoneal (ip) injection (23-30 G needle) at 10 mL/kg. Selection of each test chemical and dose was based on target organ, chemical class and previously established LD50s. The highest dose chosen for a particular dose-range represented 1/3 or 33% of the established LD50 for a particular chemical (i.e. if the MSDS LD50 is 100 mg/kg, then the initial highest dose for that chemical's dose-response study would be 33 mg/kg). This approach was approved previously in a terminated (JUN 06) metabonomic Air Force protocol (F-WA-2003-0074-A). The goal of the present experiment is to prevent any of the animals from experiencing any pain or distress from the highest exposure dose. However, animals must exhibit significant clinical and histopathological endpoints to validate potential biomarker identification by the various biotechnologies described above being utilized in this protocol. If the highest dose of a particular chemical did not produce any significant clinical or histopathological effects, the highest dose was increased 2-fold and the dose-response study repeated. Although it is possible that this process may be repeated a number of times, the highest dose tested never equaled or exceeded the established LD50 for any chemical tested. Any animal showing signs of distress or discomfort due to exposure, after consultations with the Attending Veterinarian, were euthanized. Generally, chemicals were added via protocol amendments.

### 2.3. Plasma Biochemistry

Rat blood was collected pre-dose, 24 hours post-dose and at the time the animals were euthanized. Plasma was separated from the cellular components by centrifugation and frozen at minus 80°C until analyzed. Animal plasma samples were analyzed for aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALKP), urea nitrogen (BUN), creatinine (CREA) and total protein (TP) activities using a Vet Test (Westbrook, ME) clinical chemistry analyzer. All clinical chemistry assays were performed in accordance with the manufacturer's protocols for this clinical chemistry analyzer.

### 2.4. Histopathology

Target tissue (i.e. kidney) was collected at sacrifice, fixed in 10% formalin and processed in accordance with accepted pathological protocols. Briefly, tissues were embedded in paraffin, sliced and stained with hematoxylin and eosin. Sections were evaluated by a staff veterinarian pathologist by light microscopy for mineral corpora amylacea, nephropathy, suppurative/mixed inflammation, tubular eosinophilic fluid and pyelonephritis. Severity was graded on a scale of one to four with one being minimal and four being severe.

### 2.5 Data Analysis

Clinical chemistry data was analyzed using one-way ANOVA with pair wise comparison to vehicle control with Dunnett's post hoc test Type I error level held at $p < 0.05$. Histopathology

data was statistically analyzed using paired comparisons of severity scores between the dosed and the control groups using Mann-Whitney Rank Sum Test.

## 3. RESULTS AND DISCUSSIONS

### 3.1. Histopathologic Analysis

Frozen sections of the kidney were evaluated for necrosis and graded on a 1-4 scale (1 being minimal and 4 being marked or severe) on eight morphological features: tubular eosinophilic fluid, mineral corpora amylacea, nephropathy, mixed inflammation, suppurative inflammation, pyelonephritis, dilated lymphatic leukocytosis, and papillary necrosis.

#### 3.1.1. Amphotericin B (AmpB) Treatment

No toxicity was observed with any of the AmpB doses tested as determined by clinical chemistry analysis. Results of histopathologic analysis of the kidney samples from the AmpB study were also inconclusive because it was observed that there were deposits of AmpB on the surface of a number of different organs (Figure 3). These results indicated that AmpB precipitated out of solution following ip injection and failed to be distributed systemically in a solubilized form. It was thus decided that no additional analysis should be performed with this chemical agent.

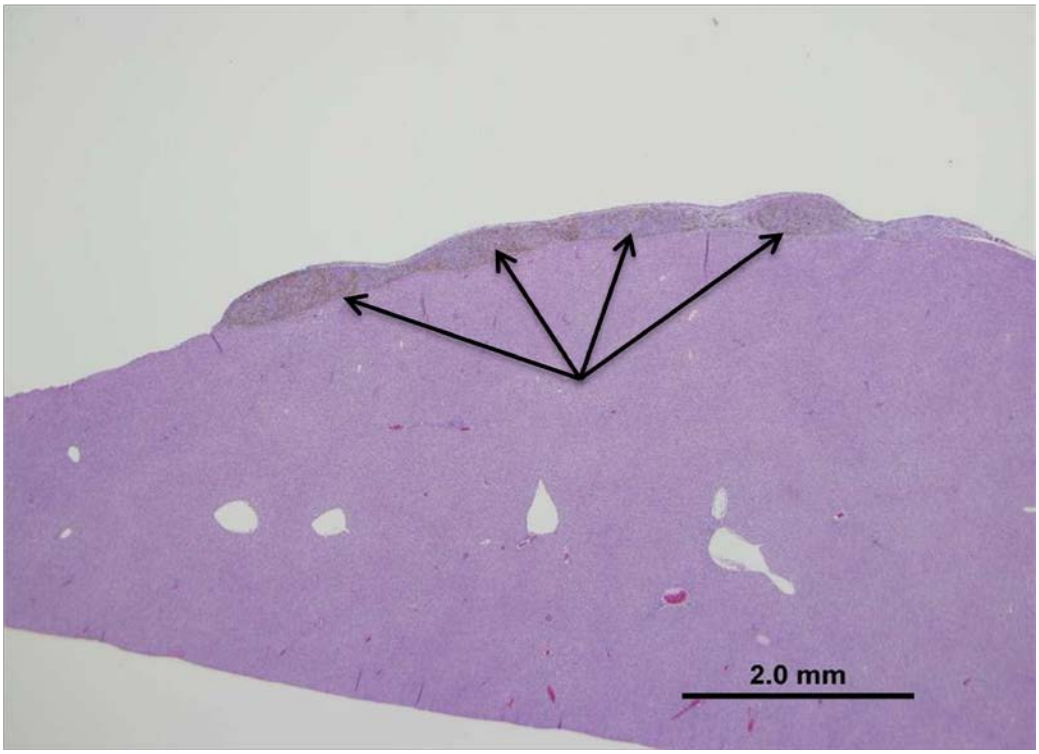


**Figure 3: Example of AmpB precipitation on the surface of the liver.**

*Haematoxylin- and eosin-stained liver section (x 20 mag) from AmpB (50 mg/kg ip) treated F 344 rats showing deposits of AmpB on organ tissue (arrows) at 4 days post exposure.*

### 3.1.2. Hippuric acid (HPA) Treatment

There were no histological lesions in the kidney that could be definitively attributed to HPA administration (data not shown). The presence of minerals (corpora amylacea) within renal tubules was noted in some control and HPA-treated animals. Since this was a common background lesion in the kidney of rats, both control and treated, it was therefore considered to be unrelated to HPA treatment. Marked superlative inflammation with bacterial colonies was observed in multiple tissues of one animal (1000 mg/kg) and appeared to be due to complications from improper venous catheter placement into the right atrium resulting in a showering of thrombotic emboli.

### 3.1.3. D-Serine Treatment

The results of histopathologic analysis of the kidney samples 96 hr after D-serine treatment are shown in Table 1. Progression of renal tubular alterations (nephropathy) with moderate to significant histopathological severity scores was observed in the kidney tissues from all animals exposed to 200 and 500 mg/kg D-serine. Specific changes included cortical and medullary tubular ectasia, attenuation and regeneration of tubular epithelial cells, thickening of tubular basement membranes, and intratubular eosinophilic protein fluid (Figure 4). However, no significant effects were observed at these two doses with respect to other kidney histopathologic endpoints. Despite that one animal from each of the three dose groups of 5, 200 and 500 mg/kg showed signs of pyelonephritis, overall the effects of D-serine exposure up to 50 mg/kg did not produce any histopathologic findings in the kidney. Control animals and treated animals exposed up to 50 mg/kg D-serine exhibited mild severity with respect to kidney mineral corpora amylacea. As discussed above, this was considered unrelated to the D-serine exposure.

**Table 1: Kidney Tissues Histopathology Analysis 96 Hours after D-Serine Exposure**

| Dose (mg/kg) | Control | | | | | 5 | | | | | 20 | | | | | 50 | | | | | 200 | | | | | 500 | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Animal # | 1 | 2 | 3 | 4 | 5 | 1 | 2 | 3 | 4 | 5 | 1 | 2 | 3 | 4 | 5 | 1 | 2 | 3 | 4 | 5 | 1 | 2 | 3 | 4 | 5 | 1 | 2 | 3 | 4 | 5 |
| Tubular eosinophilic fluid | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 |
| Mineral corpora amylacea | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Nephropat hy | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 |
| Inflammation, mixed | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Inflammation, suppurative | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Pyelonephritis | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 3 | 0 | 0 | 0 | 0 | 2 | 0 |
| Dilated lymphatics leukoc | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

0 = No abnormal findings; 1 = Minimal; 2 = Mild; 3 = Moderate; 4 = Marked/Severe

= Significantly different from Control ($P < 0.008$).

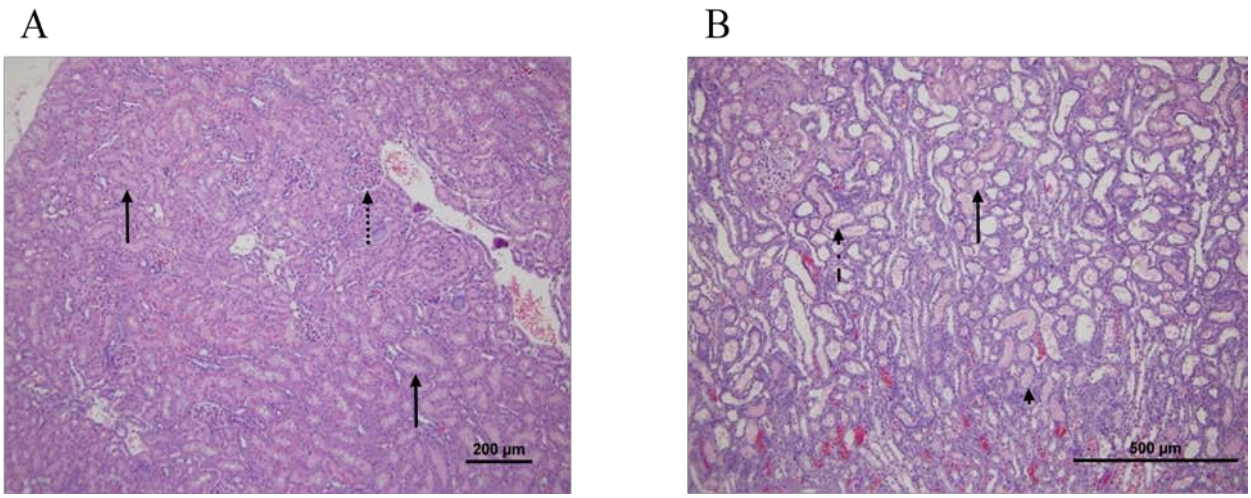


**Figure 4: D-Serine Histopathology**

*Haematoxylin- and eosin-stained kidney sections (x 100 mag) from (A) control and (B) D-serine (500 mg/kg ip) treated F 344 rats showing (A) control proximal tubules and glomerulous (dashed arrow) and (B) tubular basophila (arrowhead) with multifocal tubular dialation (solid arrow) and necrotic tubules (broken arrow) at 4 days post exposure.*

### 3.1.4. Puromycin (PUR) Treatment

Histopathologic analysis of the kidney tissues obtained on day 7 after PUR treatment revealed mild lesions in the animals dosed with 150 mg/kg of PUR. Histological changes noted include multifocal mild dilation of kidney tubules that were occasionally lined by attenuated or flattened tubule epithelial cells. Mitotic figures that were present in cells lining these tubules likely represented regeneration of damaged tubule epithelial cells (

Table 2). Protein casts present in some tubules were characterized by eosinophilic, hyaline material filling tubular lumens (Figure 5). Although there was no evidence of damage to the glomerular podocytes, slight segmental thickening was observed within glomerular tufts.

**Table 2: Kidney Tissues Histopathology Analysis 168 Hours after PUR Exposure**

| Dose (mg/kg) | Control | | | | 5 | | | | | 25 | | | | | 75 | | | | | 150 | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Animal #** | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 4 | 5 | 1 | 2 | 3 | 4 | 5 | 1 | 2 | 3 | 4 | 5 | 1 | 2 | 3 | 4 | 5 |
| Tubular Cell Degeneration | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Tubular Cell Eosinophilic Droplets | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 |
| Mineral Corpora Amylacea | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Nephropathy | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 2 | 2 | 2 | 2 |
| Inflammation, mixed | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Inflammation, suppurative | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Pyelonephritis | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Tubular Cast Formation | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Dilated Lymphatics, Leukocytosis | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

0 = No abnormal findings; 1 = Minimal; 2 = Mild; 3 = Moderate; 4 = Marked/Severe

= significantly different ($p < 0.0001$). Paired comparisons between the dosed and the control groups were performed using Mann-Whitney Rank Sum Test.

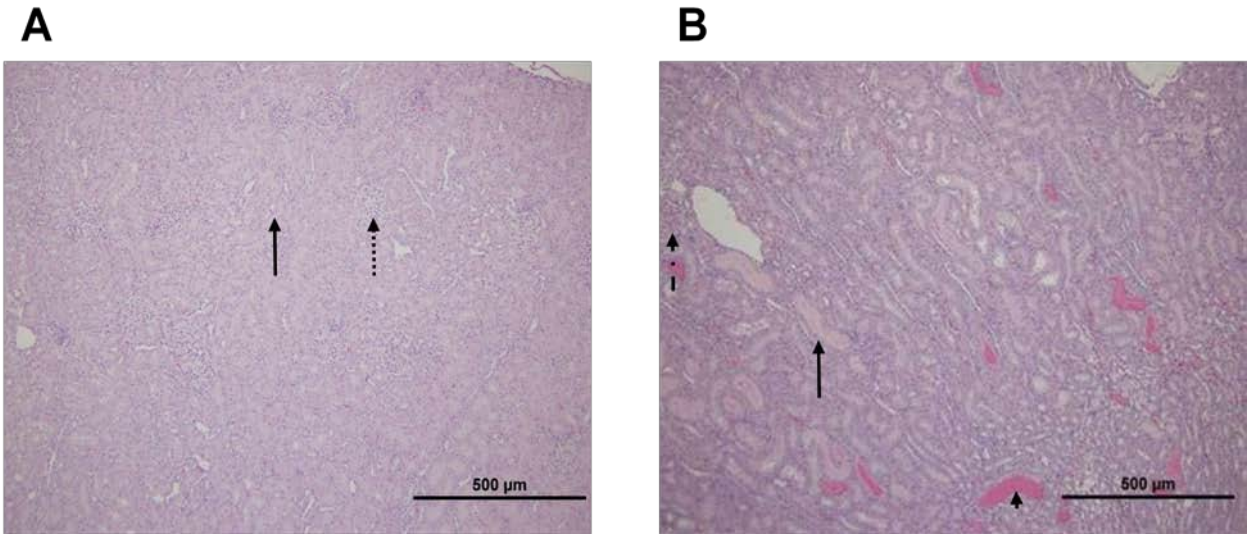


**Figure 5: Puromycin Histopathology**

*Haematoxylin- and eosin-stained kidney sections (x 100 mag) from (A) Control and (B) Puromycin (150 mg/kg ip) treated F 344 rats showing (A); control proximal tubules (solid arrow) and glomerulous (dashed arrow) and (B); tubular multifocal tubular dialation (solid arrow) filled with hyalinized proteinaceous fluid (arrowhead) and thickening glomerular tufts (broken arrow) at 7 days post exposure.*

### 3.2. Clinical Chemistry

Blood samples were collected from control and treated animals at 24 and 96 (or 168; PUR and AmpB) hours after treatment. Plasma levels of total bilirubin (TBIL), creatinine

(CREA), urea nitrogen (BUN) and total protein (TP) were measured to assess treatment-related nephrotoxicity.

### 3.2.1. AmpB Treatment

While several results were significantly different in AmpB-treated animals from control groups on day 7 post-treatment, all values were well within their respective normal ranges (Table 3).

**Table 3: Effect of AmpB Treatment on Selected Serum Chemistry Parameters**

| Treatment (mg/kg) | TBIL (mg/dL) | | CREA (mg/dL) | | BUN (mg/dL) | | TP (mg/dL) | |
|---|---|---|---|---|---|---|---|---|
| | 24hr | 168hr | 24hr | 168hr | 24hr | 168hr | 24hr | 168hr |
| 0 | 7.6±4.5 | 6.7±1.6 | 0.3±0.1 | 0.2±0.1 | 14±1.2 | 14.2±1.1 | 5.5±0.4 | 6±0.2 |
| 1 | 8.8±5.2 | 3.4±1.9* | 0.3±0.1 | 0.3±0.1 | 13±0.8 | 11.8±1.3* | 5.8±0.2 | 5.6±0.3* |
| 5 | 10.3±1.9 | 3±1.1* | 0.4±0.1 | 0.1±0.1 | 13±1.2 | 12.8±1.3 | 5.5±0.1 | 5.8±0.2 |
| 10 | 6.9±4 | 6.6±2.5 | 0.3±0.1 | 0.1±0.1 | 13.5±0.6 | 12.8±1.8 | 5.3±0.3 | 6±0.3 |
| 25 | 2.3±1.2* | 3.3±1.5* | 0.3±0.1 | 0.1±0.1 | 14±1 | 12.6±0.5* | 5.8±0.6 | 5.9±0.1 |
| 50 | 7.7±2.2 | 5.1±2.3 | 0.3±0 | 0.3±0.1 | 12.6±1.1 | 14.2±1.6 | 5.4±0.4 | 6.1±0.2 |

*Significantly different than control ($p < 0.05$)

*Plasma from blood samples collected at 24 and 168h after AmpB treatment were used in this analysis. Kidney enzymes include: total bilirubin (TBIL), creatinine (CREA), urea nitrogen (BUN), and total protein (TP).*

### 3.2.2. HA Treatment

There were no significant differences in the serum chemistries between control and HA treated animals on day 4 post-treatment (data not shown).

### 3.2.3. D-serine Treatment

The results of clinical chemistry analysis indicate that only rats in the 200 and 500 mg/kg dose groups showed a significant decrease in TBIL 24 hours following exposure compared to the control group, while the levels of CREA and BUN were significantly increased (Table 4). However, no significant change in TBIL was observed in any dose groups at the termination of the study (96 hours post-dosing). The CREA level in the rats dosed with 200 mg/kg D-serine returned to normal by 96 hours. In contrast, the level of BUN showed persistent elevation even at terminal sacrifice, although a small reduction compared to the 24 hr level was observed. On the other hand, rats exposed to 500 mg/kg D-serine exhibited elevated CREA and BUN that

remained significantly increased throughout the entire course of the study (Table 4). In fact, animals in this dose group showed the highest BUN level at 96 hours post-treatment. No significant change in TP was observed in any dose group.

**Table 4: Effect of D-Serine Treatment on Selected Serum Chemistry Parameters**

| Treatment (mg/kg) | TBIL (mg/dL) | | CREA (mg/dL) | | BUN (mg/dL) | | TP (mg/dL) | |
|---|---|---|---|---|---|---|---|---|
| | 24h | 96h | 24h | 96h | 24h | 96h | 24h | 96h |
| 0 | 5.5±1.3 | 1.6±0.8 | 0.3±0.1 | 0.5±0.1 | 15±1.6 | 17.8±1.9 | 6.1±0.1 | 6.7±0.3 |
| 5 | 5.4±2.9 | 1.9±0.8 | 0.4±0.1 | 0.4±0 | 13.8±2.4 | 18±1.2 | 5.9±0.3 | 6.7±0.2 |
| 20 | 4.1±0.2 | 2.2±1.1 | 0.4±0.1 | 0.4±0 | 16.3±1.5 | 16.8±1.8 | 6±0.2 | 6.7±0.2 |
| 50 | 5±1.4 | 1.2±1.2 | 0.4±0 | 0.4±0 | 15.3±1.3 | 16.2±1.9 | 6±0.3 | 6.5±0.3 |
| 200 | *3.5±.7 | 1.5±0.7 | *1.6±.3 | 0.7±0.2 | *7.7±5.1 | *7.8±14.2 | 6.2±0.3 | 6.7±0.3 |
| 500 | *3.2±.5 | 1.7±0.4 | *1.7±.2 | *1.2±.2 | *5.6±4.2 | *6.5±3.4 | 5.8±0.3 | 7.1±0.4 |

*Significantly different than control ($p < 0.05$)

*Plasma from blood samples collected at 24 and 96h after D-serine treatment were used in this analysis. Kidney enzymes include: total bilirubin (TBIL), creatinine (CREA), urea nitrogen (BUN), and total protein (TP).*

### 3.2.4. PUR Treatment

Four of five animals treated with PUR at 300 mg/kg died within 24 hours, probably due to severe nephrotoxicity. The remaining animal from this dose group was not included in this study. Statistical analysis of the clinical chemistry results indicated that PUR treatment at 150 mg/kg failed to significantly alter the level of CREA suggesting the absence of marked renal dysfunction (

Table 5). On the other hand, the levels of BUN in rats treated with 75 and 150 mg/kg were significantly increased compared with the low-dose treatment groups (i.e. 5 and 25 mg/kg) 24 hours after PUR exposure. One of the five rats treated with 150 mg/kg PUR showed persistent BUN elevation on day 7, in contrast to the fact that the BUN levels of other animals in the same group, as well as the animals treated with 75 mg/kg PUR, had returned to normal on day 7. TP levels were significantly decreased in the 75 and 150 mg/kg treatment groups compared with the control and the low-dose groups 24 hours post-dosing, a finding that has been previously observed in PUR-induced nephrotic syndrome (Pedraza-Chaverrí et al, 1990; Pedraza-Chaverrí et al, 1993). While the TP level of the animals in the 75 mg/kg treatment

group showed recovery on day 7, the animals dosed with 150 mg/kg of PUR showed a persistent decrease in the TP level.

**Table 5: Effect of PUR Treatment on Selected Serum Chemistry Parameters**

| Treatment (mg/kg) | CREA (mg/dL) | | BUN (mg/dL) | | TP (g/dL) | |
|---|---|---|---|---|---|---|
| | 24h | 168h | 24h | 168h | 24h | 168h |
| 0 | 0.4 ± 0.1 | 0.4 ± 0.1 | 14.3 ± 1.4 | 17.3 ± 1.9 | 7.3 ± 0.6 | 6.3 ± 0.1 |
| 5 | 0.3 ± 0.1 | 0.3 ± 0.0 | 14.6 ± 1.5 | 16.4 ± 1.9 | 6.9 ± 0.4 | 6.4 ± 0.2 |
| 25 | 0.4 ± 0.1 | 0.3 ± 0.1 | 14.0 ± 1.4 | 15.4 ± 1.7 | 6.7 ± 0.1 | 5.9 ± 0.4 |
| 75 | 0.4 ± 0.1 | 0.2 ± 0.1 | 18.4 ± 2.5[A,B] | 15.0 ± 1.9 | 5.6 ± 0.4[A,B] | 5.9 ± 0.3 |
| 150 | 0.4 ± 0.0 | 0.2 ± 0.1 | 18.0 ± 1.8 [A,B] | 21.0 ± 9.5 | 5.1 ± 0.1[A,B] | 4.5 ± 0.3[C] |

[A]Significantly different than control ($p < 0.05$)
[B]Significantly different than 5 mg/kg & 25 mg/kg ($p < 0.05$)
[C]Significantly different than all doses ($p < 0.05$)

*Plasma from blood samples collected at 24 and 168 hours after PUR treatment were used in this analysis. Kidney enzymes include: creatinine (CREA), urea nitrogen (BUN), and total protein (TP).*

## 4. CONCLUSIONS

Presently, measurable endpoints of chemical toxicity can range from histopathological changes to biochemical activities of various cellular enzymes/constituents, to the expression of specific genes/proteins/metabolites, and more recently to the global gene/protein/metabolite expression profiles in target organs or biofluids. It is widely believed that gene, protein and metabolite expression changes are more accurate, reliable, sensitive, and informative as quantitative endpoints than the traditional clinical chemistry toxicity endpoints. Genomic, proteomic and metabonomic biotechnologies are ideal tools to measure biological changes resulting from toxicant exposure in the systemic scale. Understanding the cellular response to chemical exposure at the molecular level will not only facilitate the elucidation of the mechanism of chemical toxicity, but also allow accurate prediction of chemical toxicity and phenotypic outcome. Ultimately, this will lead to the identification of novel biomarkers for rapid monitoring and prediction of health hazards associated with chemical exposure, as well as pinpointing gene, protein and metabolic pathway targets for nutritional and pharmaceutical prophylactic and therapeutic intervention. The present study, unlike previous "omics" studies in the literature,

attempted to incorporate all three “omics” (genomics, proteomics and metabolomics) analyses into one experimental design.

Although the experimental design described above was found to be well suited for the metabonomics work, it was found to be inadequate for both genomics and proteomics with respect to sample collection times. The main reason for this was the fact that metabonomics experiments required continual urine sample collection over time from the same animals to monitor changes in metabolite profiles. To make these same types of measurement for either genomics or proteomics, it would require serial sacrifice of animals at appropriate time points for each of these “omics” technologies. However, both genomic and proteomic measurements made using the initial experimental design at terminal sacrifice were able to identify biomolecular profile differences between treated and control animals. The study design was later changed to address this deficiency, but no other animal studies were funded to continue this work.

Chemical selection for the study was targeted toward the kidney; one of a number of organ systems identified prior to the study as being important to impacting warfighter performance and health if degraded. Chemicals that targeted various tissue structures/functions of the kidney (i.e. proximal tubule, distal tubule, and glomerulous) were selected as a proof-of-concept validation of our “omics” dose-response analysis approach. Doses of these nephrotoxicants were found to be appropriate and were based on prior literature values where possible. However, two of the four kidney toxicants utilized in this study failed to induce kidney tissue specific toxicity. In the case of AmpB, it was discovered that the compound did not appear to be solubilized following dosing. Consequently, histopathological data indicated that the compound was found to have precipitated onto the surface of a number of different organ tissues. This finding suggests potential problems in previously published work that showed no toxicity in rats exposed to AmpB by ip injection. Hippuric acid was also found not to be toxic at the dose levels used in this study. Increasing the dose of HA produced a sharp transition in toxicity that resulted in rapid death with doses exceeding 1250 mg/kg. This was believed to be the result of an induced metabolic acidosis due to the high dose of acid. No further experiments were carried out using this compound.

Histopathology and clinical chemistry measurements are crucial when trying to correlate “omics” profile changes with associated organ-specific toxicity. The highest dose chosen for a particular dose-range represented 33% of an established LD50 for a particular chemical. The goal of the experiments was to prevent any of the animals from experiencing any pain or distress from the highest exposure dose. However, animals must exhibit significant clinical and histopathological endpoints to validate potential biomarker identification by the various biotechnologies described above being utilized in this study. If the highest dose of a particular chemical did not produce any significant clinical or histopathological effects, the highest dose was increased 2-fold and the dose-response study repeated. Although it was possible that this process may be repeated a number of times, the highest dose tested was never equal to or exceeded an established LD50. Histopathology and clinical assay determinations for the D-serine and PUR exposure studies indicated that the highest doses used only induced significant mild toxicity in the targeted tissue (Table 1 and 2; Table 4 and 5). Therefore, “omics” measurements made at these doses could be associated with clinically determined mild toxicity. This allowed for potential biomarkers of toxicity to be identified that may be predictive of

potential toxic effects at lower exposure levels. Although the initial study design did allow for potential biomarker discovery of kidney degradation from all three biotechnologies employed (genomics, proteomics and metabonomics), the optimal study design for biomarker discovery for each "omics" should be developed independently to observe changes in gene, protein and metabolite profiles at their appropriate times of induction (i.e. early gene changes to later metabolite changes).

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